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Colchicine Promotes Antigen Cross-Presentation by Murine Dendritic Cells

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TO THE EDITOR

Colchicine (COL) is a microtubule depolymerizing drug that has been widely used for the treatment of many inflammatory skin diseases, such as Behçet disease, psoriasis, palmoplantar pustulosis, Sweet's syndrome, and leukocytoclastic vasculitis (Sullivan *et al.*, 1998; Bibas *et al.*, 2005). With regard to its pharmacological mechanisms of action, COL is thought to primarily inhibit various functions of polymorphonuclear neutrophils, including mitosis, adhesion, migration, and phagocytosis (Keller *et al.*, 1984; Cronstein *et al.*, 1995; Blocker *et al.*, 1997; Jordan and Wilson, 2004). We recently reported totally unexpected pharmacological activities of COL (Mizumoto *et al.*, 2005). Briefly, COL at relatively low concentrations triggered phenotypic maturation of murine bone marrow (BM)-derived dendritic cells (DCs) as well as their production of several cytokines and chemokines. Moreover, BM-DCs pretreated with COL exhibited enhanced capacities to activate allogeneic T cells and to present a foreign protein antigen to immunologically naive CD4 T cells. Interestingly, COL pretreatment markedly improved the ability of BM-DCs to uptake FITC-conjugated dextran (DX). Upon local injection in low doses, COL promoted *in situ* maturation and mobilization of epidermal Langerhans cells and boosted both humoral and cellular immune responses in mice (Mizumoto *et al.*, 2005). These observations may appear to be rather contradictory to the conventional view that COL acts as an anti-inflammatory agent (when administered systemically at high doses). Thus, we first sought to study *in vitro* effects of COL on human DCs.

For this purpose, we employed human DC preparations generated from $CD34^+$ progenitors in the cord blood. Following 24-hour pretreatment with 3 μg/ml COL, we examined surface expression of major histocompatibility complex (MHC) class II molecules, CD83, and co-stimulatory molecules CD40, CD80, and CD86 within the $CD1a^+$ populations. COL treatment significantly elevated the expression of all tested markers of DC maturation without affecting their viability (Figure 1a).

Abbreviations: BM, bone marrow; COL, colchicine; DC, dendritic cell; DX, dextran; ER, endoplasmic reticulum; MHC, major histocompatibility complex; OVA, ovalbumin; TAP, transporter associated with antigen presentation

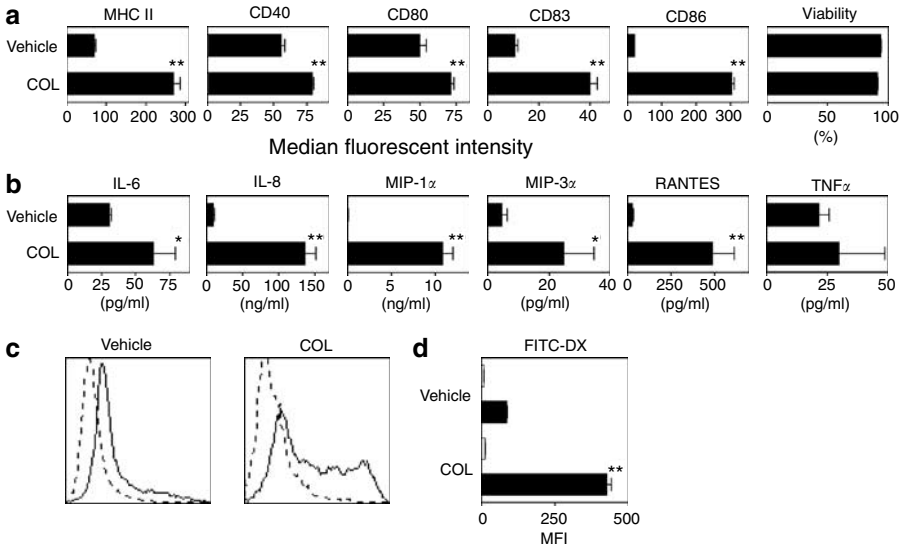

Figure 1. *In vitro* impacts of COL on human DCs. (**a**) Human DC cultures generated from CD34$^+$ progenitors purified from the cord blood were purchased from MatTek Corporation (Ashland, MA); an overwhelming majority of these DCs reportedly expresses CD1a, a marker of human Langerhans cells. Following 24-hour incubation with 3 μg/ml COL or vehicle alone, human DCs were examined for the surface expression of the indicated markers within the CD1a$^+$ populations and for cell viability by propidium iodide uptake (mean$\pm$SD, $n=3$). (**b**) The supernatants of the same human DC cultures were examined for the secretion of the indicated cytokines and chemokines (mean$\pm$SD, $n=3$). (**c**) Following 24-hour incubation with 3 μg/ml COL or vehicle alone, human DCs were examined for their capacity to uptake FITC-DX (70,000 Da molecular weight) from Sigma (St Louis, MO) at 4°C (broken lines) or 37°C (solid lines). The data shown are representative histograms for the FITC fluorescent signals within the CD1a$^+$ populations. (**d**) The data shown are the mean$\pm$SD ($n=3$) of the mean fluorescence intensity (MFI) of FITC-DX uptake of COL-treated or vehicle-treated human DCs tested at 4°C (open bars) or 37°C (closed bars). Statistically significant differences compared with the vehicle-treated control samples are indicated with asterisks (*$P<$ 0.05, **$P<$ 0.01). Each experiment was repeated at least three times to assess reproducibility.

Moreover, COL triggered robust production of IL-6, IL-8, macrophage inflammatory protein-1α, macrophage inflammatory protein-3α, and regulated on activation, normal T cell expressed and secreted chemokine (Figure 1b). However, COL failed to induce significant production of tumor necrosis factor-α. Importantly, COL pretreatment also improved the ability of human DCs to uptake FITC-DX as observed with murine DCs. Control human DCs treated with vehicle alone showed modest FITC-DX uptake at 37°C and minimal surface binding at 4°C, whereas COL-treated DCs showed an augmented endocytic capacity (Figure 1c). Quantitative analysis revealed 5-fold improvement of FITC-DX uptake after COL treatment (Figure 1d). Heterogeneity of the FITC-DX uptake profile observed in COL-treated human DC populations did not appear to reflect their states of maturation because CD86 expression was elevated uniformly in an overwhelming majority of such populations after COL treatment (data not shown).

COL treatment of murine BM-DCs augmented their capacity to internalize not only FITC-DX, but also FITC-BSA (Figure 2a). These observations may first appear to be contradictory to the general notion that DCs downregulate their endocytic capacity upon maturation (Sallusto *et al.*, 1995). Selected Toll-like receptor ligands, including lipopolysaccharides, have been reported to enhance DC endocytosis *via* causing rapid reorganization of actin cytoskeletal filaments (West *et al.*, 2004). When compared with COL in parallel, however, lipopolysaccharides induced only marginal, if any, augmentation of the uptake of FITC-DX or FITC-BSA by BM-DCs (data not shown). Our findings with COL now suggest that endocytic machinery may be regulated by the extent of microtubule polymerization in DCs. Indeed, confocal microscopic study revealed partial disruption of microtubule networks in COL-treated BM-DCs, whereas control DCs treated with vehicle alone showed well-developed microtubule formation (Figure 2b). No significant change was observed in the actin microstructures after COL treatment.

Upon incorporation of apoptotic bodies and cellular proteins derived from virally infected cells and dying tumor cells, DCs are known to present the MHC II and MHC I peptide epitopes to CD4 and CD8 T cells, respectively (Huang *et al.*, 1994; Albert *et al.*, 1998; Sigal *et al.*, 1999). The latter event, termed ''cross-presentation,'' represents an unusual antigen processing pathway in which exogenous antigens are presented in a MHC I-restricted manner to CD8 T cells (Ackerman and Cresswell, 2004). With regard to mechanisms, cross-presentation involves the fusion of endoplasmic reticulum (ER) with early phagosomes to form specialized organelles equipped with all the components required for MHC I peptide loading, such as newly synthesized MHC I molecule, transporter associated with antigen presentation (TAP), tapasin, and calreticulin. After ER–phagosome formation, maturation of ER–phagosomes through the endocytic pathway results in acquisition of lysosomal components that help the breakdown of internalized proteins. These polypeptides then are retro-transported to the cytosol for proteasome-dependent degradation, and the resulting peptides are transported back to the phagosomes *via* TAP complex for MHC I loading (Ackerman and Cresswell, 2004; Heath *et al.*, 2004; Trombetta and Mellman, 2005). Thus, we next examined the subcellular localization of exogenous antigens endocytosed by COL-stimulated DCs using FITC-DX as a probe. FITC fluorescent signals showed almost complete overlap with the blue fluorescent signals demarcating the ER compartments within 10 minutes of incubation (Figure 2c). Moreover, colocalization of FITC fluorescent signals with the red fluorescent signals demarcating the lysosomal compartments became detectable 30-minutes post-internalization (Figure 2d). We interpret these patterns to suggest

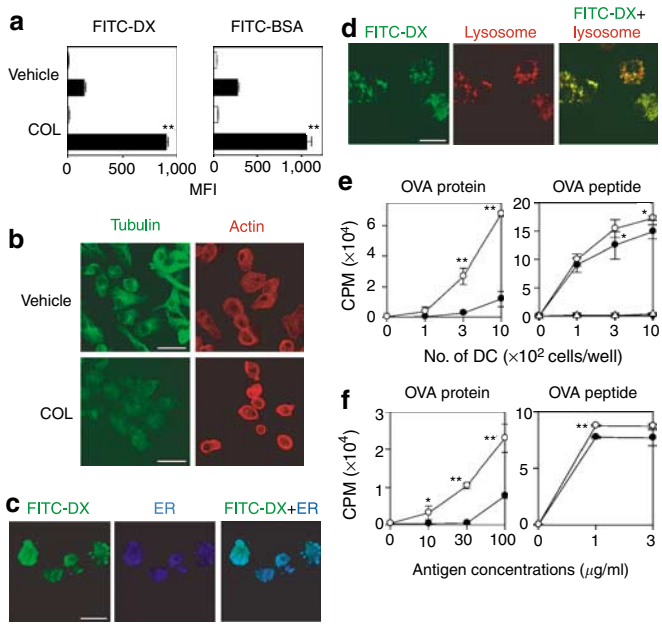

Figure 2. Enhanced antigen cross-presenting capacity by COL-treated murine DCs. (**a**) Following 24-hour incubation with 3 μg/ml COL or vehicle alone, BM-DCs propagated from BALB/c mice were examined for their capacity to uptake FITC-DX, or FITC-BSA at 4°C (open bars), or 37°C (closed bars). Samples then were examined for FITC signals (mean ± SD, $n = 3$) within the $CD11c^+$ populations. (**b**) After 24-hour incubation with 3 μg/ml COL or vehicle alone, BM-DCs were stained for tubulin (green) or actin (red), and then examined by confocal microscopy (Leica Microsystems, Wetzlar, Germany). Fluorescence was detected using a ×100/1.4 numeric aperture apochromatic objective (APO). Images shown are maximum intensity projections of the *x–y* planes generated by MetaMorph software (Universal Imaging, Downingtown, PA) and ImageJ program (NIH, Bethesda, MD). Bar = 25 μm. (**c**) Following 24-hour pretreatment with 3 μg/ml COL, BM-DCs were stained with ER-Tracker Blue-White DPX dye (blue) from Molecular Probes (Eugene, OR) and then incubated with FITC-DX (green). (**d**) The same BM-DC cultures were allowed to internalize FITC-DX first, extensively washed, and then stained for lysosome (red) with LysoTracker Red DND-99 (Molecular Probes) for an additional 30 minutes at 37°C to allow complete transfer of DX to the lysosome. Colocalization of FITC-DX with ER or lysosome was examined by confocal microscopy. All two-color images were acquired with a sequential scan mode using Leica Confocal Software. Bar = 25 μm. (**e**) Following 24-hour pretreatment with 3 μg/ml COL (open symbols) or vehicle alone (closed symbols), BM-DCs propagated from C57BL/6 mice were pulsed for 1 hour with 300 μg/ml OVA protein (left panel), 10 μg/ml $OVA_{257–264}$ peptide (circles in right panel), or PBS alone (triangles). After extensive washing, the DC samples were co-cultured at the indicated numbers with OVA-reactive CD8T cells (5×10^4 cells/well) purified from OT-I transgenic mice (kindly provided by Dr James Foreman, UT Southwestern). Data shown are ^{3}H-thymidine uptake on day 4 (mean ± SD, $n = 3$). (**f**) The above experiments were repeated by pulsing COL-treated DCs (open circles) or vehicle-treated DCs (closed circles) with OVA protein (left panel) or $OVA_{257–264}$ peptide (right panel) at the indicated antigen concentrations. After extensive washing, the DC samples (10^3 cells/well) were co-cultured with OVA-reactive CD8 T cells (5×10^4 cells/well) to measure ^{3}H-thymidine uptake on day 4. Statistically significant differences compared with the vehicle-treated DCs are indicated with asterisks (*$P < 0.05$, **$P < 0.01$).

sequential intracellular transports of the internalized probe to the phagosome–ER fusion compartments and then to the lysosome, a major antigen processing pathway for cross-presentation (Trombetta and Mellman, 2005).

The above observations implied further that COL may promote cross-presentation of exogenous antigens to CD8 T cells. In fact, COL-treated BM-DCs exhibited marked (5-fold) and significant ($P < 0.01$) improvement over the vehicle-treated control DCs in their ability to cross-present intact ovalbumin (OVA) proteins (300 μg/ml) to CD8 T cells freshly isolated from OT-I TCR transgenic mice, in which a majority of CD8 T cells recognize the $OVA_{257–264}$ peptide (SIINFEKL) presented by the MHC I molecules, H-$2K^b$ (Figure 2e). By contract, COL treatment induced only marginal (up to 1.2-fold) improvement in their ability to present the $OVA_{257–264}$ peptide (10 μg/ml) to the same OT-I CD8 T ceils. It should be noted that the extent of T-cell growth induced by COL-treated/OVA protein-loaded DCs (i.e., up to 65,000 c.p.m.) was substantial when compared to the extent observed in our positive control in which COL-treated DCs were pulsed with excessive amounts of the $OVA_{257–264}$ peptide (i.e., up to 170,000 c.p.m.). In antigen dose-dependency experiments, COL-treated DCs were found to be much more efficient than vehicle-treated DCs in their capacity to cross-present (10–100 μg/ml) OVA proteins to OT-I T cells at all tested antigen concentrations (Figure 2f). Once again, COL treatment induced no significant or only marginal improvement of their capacity to present the $OVA_{257–264}$ peptide even in a lower concentration range (1–3 μg/ml). Taken together, these observations demonstrate that COL enhances the *in vitro* ability of murine BM-DCs to cross-present a protein antigen to CD8 T cells.

To recapitulate the essence of our new observations, COL was found to promote phenotypic maturation and cytokine/chemokine production by human DCs. We also found that COL markedly augments the ability of murine BM-DCs to cross-present exogenous protein antigens to CD8 T cells. At least two key questions, however, remain to be addressed experimentally. Does COL treatment also enhance antigen cross-presentation by human DCs? Can we use COL (or COL-pretreated DCs) to promote cross-presentation of tumor-associated antigens or microbial antigens *in* vivo? Nevertheless, this study suggests a previously unrecognized pharmacological activity of COL, which should be taken into consideration when administering COL to patients with inflammatory skin disorders.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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A Mechanistic Study of δ-Aminolevulinic Acid-Based Photodynamic Therapy for Cutaneous Leishmaniasis

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TO THE EDITOR

Photodynamic therapy (PDT) is based on the concept that a certain photoactivatable compound, called a photosensitizer, can be excited by light of the appropriate wavelength to generate cytotoxic singlet oxygen and free radicals (Hasan *et al.*, 2006). Although earlier reports of PDT involved microbiological applications (Raab, 1900), clinically PDT has been developed most extensively for oncologic and ophthalmologic applications. Recent clinical outcomes of PDT using δ-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX), termed ALA-PDT, in patients with cutaneous leishmaniasis (CL) have been promising (Enk *et al.*, 2003; Gardlo *et al.*, 2003, 2004; Asilian and Davami, 2006), but far from being curative.

ALA by itself is not a photosensitizer, but is processed to PpIX in the heme biosynthetic pathway where it serves as a biological precursor of PpIX. It is known that *Leishmania amazonensis* parasites are deficient in seven out of eight enzymes in the heme biosynthetic pathway (Sah *et al.*, 2002). Although *Leishmania major* (*L. major*) have not been studied for this enzyme deficiency, as they require extracorporeal supplementation with PpIX or hemin as a growth factor *in vitro* (Steiger and Steiger, 1976; Chang and Chang, 1985), it is reasonable to assume that they may also be deficient in these enzymes, and that they cannot produce PpIX from ALA. Therefore, the reported efficacy of ALA-PDT for CL is somewhat intriguing.

Encouraged by clinical reports, and with a view to increasing the efficacy of ALA-PDT for CL, we performed a series of *in vitro* and *in vivo* experiments to unravel the underlying mechanisms associated with this clinical response. In an attempt to better understand the basis of ALA-PDT for CL, we identified several key questions that are addressed here: first of all, how does ALA-PDT work for CL? At the cellular level, is ALA-PpIX produced by *Leishmania* or by host cells, or by both? Do *Leishmania* parasites take up ALA-PpIX from host cells?

We studied a series of cellular events in *Leishmania* parasites and in infected immortalized macrophages (J774.2) during ALA-PDT. The amount of PpIX in the stationary phase of a

Abbreviations: ALA, δ-aminolevulinic acid; ALA-PDT, photodynamic therapy with 5-aminolevulinic acid-induced protoporphyrin IX; ALA-PpIX, 5-aminolevulinic acid-induced protoporphyrin IX CL, cutaneous leishmaniasis; PDT, photodynamic therapy; PpIX, protoporphyrin IX